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NUTRITION REVIEWS

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NUTRITION REVIEWS

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PLASMA IRON: ITS TRANSPORT AND SIGNIFICANCE

Iron essential to man for use in the synthesis of his hemoglobin, myoglobin, cytochromes and iron-containing catalysts is derived from his diet and made available by an iron-specific transport mechanism in the blood plasma which carries the iron absorbed from the gastrointestinal tract to the appropriate cells and tissues. This iron transport system is responsible for disposition not only of the absorbed iron but of the iron derived from the breakdown of hemoglobin and from the storage organs as well. The magnitude of the physiologically conditioned movement of plasma iron can be appreciated from the fact that 25 to 30 mg. of iron are turned over, under normal conditions, every twenty-four hours. Seventy-five per cent of this turnover iron is accounted for by the daily requirements of hemoglobin synthesis alone and is provided by the iron freed in daily hemoglobin breakdown. Thus, a major responsibility of the plasma iron transport system is to retain the iron released from the hemoglobin of blood cells, normally catabolized outside the bone marrow, and to make it available to the erythropoietic tissues for new blood cell production.

Siderophilin (A. L. Schade and L. Caroline, *Science* **104,** *340 (1946)*) (transferrin (C.-B. Laurell, *Blood* **6,** *183 (1951)*); beta$_1$-metal-combining (pseudo) globulin (D. M. Surgenor, B. A. Koechlin, and L. E. Strong, *J. Clin. Invest.* **28,** *73 (1949)*) is the protein component of plasma which functions as the iron carrier in the plasma iron transport system. Each molecule of siderophilin binds two atoms of iron, in the ferric state, in a complex with two molecules of carbonate. In one twenty-four-hour period, the total amount of iron bound to siderophilin is turned over seven to eight times. This circumstance results from the fact that while, for example, there is sufficient siderophilin in the plasma of a 70 kg. man (about 9 g.) to carry 11 to 12 mg. of iron, only one third of this available siderophilin is iron-saturated. Hence, 3.5 to 4 mg. of iron are transported at any moment in the circulating blood to provide for the 25 to 30 mg. daily metabolic iron requirement.

Although the siderophilin-iron-carbonate complex in plasma is chemically stable at the pH of blood, physiologically the iron is apparently readily available to satisfy metabolic needs. Since a lowering of the pH to 7.0 and below dissociates the iron-siderophilin complex, and since the reducing conditions of plasma lead to conversion of the ferric iron to the more soluble ferrous form, the rapid exchange of iron from the complex to the iron-requiring tissues (and vice versa) may be brought about by equilibrium shifts resulting from altered physiologic states affecting these conditions. In this connection it is suggested that, although evidence to date shows that ascorbic acid per se does not affect intermediate iron metabolism (L. E. Tötterman, *Acta med. Scandinav., Suppl. 230, 1 (1949)*), the ascorbic acid of the blood may function as the normal ferric-to-ferrous iron reductant and play a major role in effectuating the rapid equilibration between siderophilin and the iron-metabolizing tissues or stores.

The circumstances of the discovery of siderophilin as the iron-binding component of blood plasma illustrate a case of a nutritional study leading to the elucidation of a problem of lively concern to investigators of another discipline, hematology, as well as to the provocation of some hitherto unasked questions. The initial nutritional problem arose from an observation that

raw egg white, when added to a broth culture of dysentery bacilli, inhibited their growth. Addition of yeast extract to the inhibited culture permitted growth to recur. Systematic addition of vitamins, amino acids, and minerals known or suspected to be present in the yeast extract to the inhibited cultures finally revealed iron to be the needed element. Investigation of the relationship between egg white and iron then disclosed that some protein component was binding iron to the extent of 20 micrograms per milliliter of egg white and thus effecting deprivation of the iron in the broth medium required by the dysentery bacilli for their nutrition (A. L. Schade and L. Caroline, *Science* **100,** *14 (1944)*). Through collaborative effort (G. Alderton, W. H. Ward, and H. L. Fevold, *Arch. Biochem.* **11,** *9 (1946)*), the effective protein component was determined to be conalbumin, representing 15 per cent of the total egg white proteins. Now, out of these facts many questions arise concerning the iron nutrition and development of the chick embryo, the function of conalbumin therein, the mechanism for the production and laying down by the hen of an iron-avid proteinous egg white surrounding an iron-rich yolk, the kind of iron transport system of the chick and the mature animal, etc. Partial answers to some of these questions have appeared but, on the whole, the field of inquiry is virgin.

When egg white, so intimately associated with a developing embryo, showed such affinity for iron, it seemed not amiss to look for a protein with similar properties in blood plasma. Preliminary attempts with blood serum to effect inhibitions of growth of bacillary dysentery cultures were unsuccessful, although positive indications of the looked-for component were given by the response in the increase of light absorption at wave length 465 millimicrons by a previously acid-dialyzed serum sample following iron addition. Upon request to E. J. Cohn, and through his generosity and that of his collaborators, the several blood fractions were made available and tested for their iron-binding capacity both by the bacterial growth-inhibition test and the quantitative colorimetric test developed during the iron-egg white investigations. Only fraction numbered IV:3,4 showed activity. As soon as it was realized that a component of this fraction alone was responsible for the demonstrated iron affinity, the Harvard group began to subfractionate IV:3,4. As a result of collaborative testing of the several fractions isolated, subfraction IV:7 emerged as the richest in the beta$_1$-globulin from which crystalline siderophilin was later prepared.

The identification of siderophilin as the iron-binding component of blood plasma culminated twenty years of investigation by many workers (C.-B. Laurell, *Pharm. Rev.* **4,** *371 (1952)*) of the nonhemoglobin-plasma iron and resulted in a resurgence of interest in its significance in health and disease. Along with determinations of the concentrations of bound plasma iron found in both normal males and females, the concentrations of iron-free siderophilin have likewise been estimated. While human males show approximately 120 to 140 micrograms per cent of bound plasma iron, females have a lower level of 90 to 120 micrograms per cent. The total iron-binding capacity of the plasma, on the other hand, is roughly the same for both sexes, namely, 300 to 360 micrograms of iron per 100 ml. of plasma. Of particular interest is the fact that the amount of bound plasma iron exhibits a diurnal variation (K. Høyer, *Acta med. Scandinav.* **119,** *577 (1944)*). The extent of variation can be as great as 60 micrograms per cent over a twenty-four-hour period, with the lowest value recorded two hours following retirement for sleep and the highest after an additional five to seven hours. Correlations with other normal physiologic variations remain to be developed. A second fact of interest is that toxicity reactions from intravenous injections of readily dissociable iron compounds

depend upon the concentration of available iron-free siderophilin in the circulating blood. When the amount of free iron exceeds the unsaturated iron-binding capacity, iron intolerance ensues.

Deviations from the normal values of both the amount of bound iron and the total iron-binding capacity of plasma are of great importance in the diagnoses and the treatment of a number of disease states. Characteristic ratios of bound plasma iron to the unsaturated iron-binding capacity are known for such diseases as polycythemia vera, hemolytic and pernicious anemias, acute and chronic infections, hemochromatosis and transfusion hemosiderosis, acute hepatitis, cirrhosis, malignancy, and chronic iron deficiency, etc. A close check on the plasma iron levels as well as the total iron-binding capacities during therapy for several of the disease conditions permits useful judgments to be made of the progress of treatment. In the treatment of pernicious anemia, for example, it is possible to determine the efficacy of a hematinic agent by noting the decrease in bound plasma iron consequent upon administration of the agent. In addition, use of radioactive iron as tracer, when combined with plasma iron determinations, has proven valuable in many hematologic investigations.

While the reported data on bound plasma iron are fairly extensive for the normal and pathologic conditions, data on the concentration of iron-free siderophilin in any given plasma sample are relatively sparse. This fact reflects primarily the availability, until recently (A. L. Schade, J. Oyama, R. W. Reinhart, and J. R. Miller, *Proc. Soc. Exp. Biol. Med.* **87,** *443 (1954)*), of more simple and reliable methods for the determination of bound iron of plasma than for that of its total iron-binding capacity. Since it is now possible to obtain both values with such ease and rapidity that on one milliliter or less of plasma within an hour's time the desired data can be had, new findings of importance are anticipated. Not only is this the case for human physiologic studies but, more particularly, animal investigations of iron metabolism should be stimulated. From a nutritional point of view, increases in siderophilin concentration of the plasma, found in cases of iron deficiency and especially in instances of bacterial infection recently observed in rabbits where its concentration increased over three-fold, may be of significance for metabolic studies of vitamin and amino acid nitrogen requirements.

Although the role of siderophilin as the iron carrier in plasma has dominated interest in this blood component, it is not at all certain that its physiologic function is restricted to this purpose. By very reason of the circumstances, already noted, under which it was first discovered, siderophilin has a claim to further investigation as an important agent in host resistance to microbial infection.

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EFFECT OF THYROID HORMONE ON ALBUMIN TURNOVER IN MYXEDEMA

The disease myxedema results from inadequate function of the thyroid gland. It is associated with decreased basal metabolic rate, slow pulse, dry skin, accumulation of excessive quantities of fluid in tissues of the body and elevation of cholesterol concentration in blood serum. The administration of thyroid substance to such patients causes mobilization and excretion of fluid, negative nitrogen balance and elevation of the basal metabolic rate.

E. Schwartz (*J. Lab. Clin. Med.* **45,**

340 (1955)) has published the results of experiments which indicate that the fluid accumulations in myxedema are rich in protein, with a decreased rate of degradation of albumin and prolonged half-life of this protein contributing to retention of protein and water. Two men, aged 53 and 60, with myxedema were studied. In both patients the basal metabolic rate was low and there was no measurable uptake of radioiodine in the thyroid gland of either patient. The experimental approach was one which determined the miscible pool of albumin before and after institution of therapy with desiccated thyroid substance and permitted estimation of the rate of turnover of albumin under both circumstances. To accomplish these purposes iodinated human serum albumin, containing approximately 300 microcuries of the iodine isotope, I^{131}, was diluted in saline and injected intravenously. A specimen of blood plasma taken ten minutes after injection of the labeled albumin permitted estimation of the plasma volume. The radioactivity persisting in association with plasma albumin was determined at daily intervals thereafter. When the labeled human albumin is metabolically degraded, iodine is freed and promptly excreted in the urine. Therefore, determination of the I^{131} in twenty-four-hour specimens of urine permitted determination of the quantity of albumin split daily. At longer intervals of several days the plasma proteins were separated by fractionation with sodium sulfite and the specific activity of the iodine tag per milligram of albumin was determined. All radioactive counts as determined were corrected to counts at the time of injection utilizing tables denoting rates of physical decay of the isotope.

The labeled albumin injected is after several days evenly distributed in the body pool of miscible albumin. The pool is composed of quantities of the protein circulating in plasma and present in extravascular sites. The procedures utilized determined not only the size of the total miscible pool but plasma albumin and extravascular albumin as well. The data indicated that prior to treatment with thyroid there was substantial increase in the miscible pool of albumin in these patients with myxedema. Administration of 128 mg. of desiccated thyroid daily for ten weeks resulted in a decrease of 14 per cent in the quantity of miscible albumin in one patient and of 27 per cent in the other patient. All of the change was in the albumin in extravascular sites. Other information indicated that the half-life of albumin is increased in myxedema. In one patient the half-life determined by several methods of calculation was 27 days prior to and 17 days after therapy. The implications of these observations are that the thyroid gland has a direct role in the metabolism of albumin and that one function of the gland is to control the rate of degradation of this protein. In states of thyroidal hypofunction albumin accumulates because it is split less rapidly, the excess finding its way into extravascular supplies of the protein, where it permits the imbibition and retention of water in tissues, producing the characteristic swelling or edema.

The deductions drawn relate to findings in but 2 patients. However, the changes observed were relatively large and seemed to result from institution of therapy with thyroid. The treatment given produced other changes indicating correction of the hypothyroid state. In both patients the basal metabolic rate was increased to normal levels and the serum cholesterol concentration was reduced. Values recorded for albumin half-life and for total exchangeable albumin after treatment are reasonably close to those recorded by other authors.

Although it seems to have been established that degradation of albumin is impaired in myxedema, the mechanism for accumulation of protein in extravascular sites has not been elucidated. The author considers mechanisms which depend upon changes either in

capillary permeability or in the metabolism of protein. The second of these mechanisms is probably more important than the first, since the administration of thyroid substance to animals and patients with normal thyroidal function or with hypothyroidism has been found to induce negative nitrogen balance and increased rates of protein breakdown (F. Albright, W. Bauer, and J. C. Aub, *J. Clin. Invest.* **10,** *187 (1931)*; F. B. Byrom, *Clin. Sci.* **1,** *273 (1934)*).

The observations of Schwartz (*loc. cit.*) are of interest and importance because they afford description of a major metabolic aberration resulting from inadequate function of the thyroid gland. They demonstrate important relationships between the hormone of this gland and protein metabolism.

ALCOHOL, NUTRITION AND LIVER BLOOD FLOW IN MAN

Cirrhosis of the liver in experimental animals and in man is well known to follow prolonged consumption of an inadequate diet, particularly when it is deficient in protein, including methionine and choline. The problem is more complicated in man than in animals because of the possibility that chronic infections, malaria and alcoholism may either condition the nutritional deficiency or directly damage the liver. One of the most prominent and at the same time earliest lesions found in the livers of both animals and patients is fatty infiltration, which H. P. Himsworth ("The Liver and Its Diseases," *2d edition, Harvard University Press, Cambridge (1950)*) has suggested might interfere with hepatic blood flow, and diminish nutrient supply, particularly centrolobularly, resulting in loss of hepatic substance in this area with subsequent fibrosis.

The development of the method for determining hepatic blood flow in man by S. E. Bradley, F. J. Ingelfinger, G. P. Bradley, and J. J. Curry (*J. Clin. Invest.* **24,** *890 (1945)*) has made it possible to measure alterations of this function under a variety of clinical conditions. In this way, A. I. Mendeloff (*Ibid.* **33,** *1298 (1954)*) studied the effect of intravenous alcohol on blood flow through the normal liver, and B. J. Kessler, J. B. Liebler, G. J. Bronfin, and M. Sass (*Ibid.* **33,** *1338 (1954)*) determined the blood flow in alcoholics with fatty liver disease.

In the first study 5 young adults without evidence of malnutrition, liver disease, or other abnormalities except either gastric or duodenal ulcers were given an intravenous infusion of alcohol in saline following a control period during which saline was given alone. Hepatic vein catheterization was carried out during both periods, and the bromsulfalein infusion for measuring hepatic removal was administered continuously by adding it to both the saline and saline-alcohol infusions. In this way it was possible to measure the "estimated hepatic blood flow" (in milliliters per minute) during the control period and the periods when alcohol was given (ranging from fifty to eighty minutes). A distinct increase in the hepatic blood flow (from 28 to 58 per cent above the control value) occurred with alcohol in each instance.

In addition to hepatic blood flow, glucose, lactate, pyruvate, pepsinogen, and in the alcohol period, alcohol and acetaldehyde, were measured in the peripheral and hepatic vein blood to show the extent of hepatic removal or production of these substances. Livers in 4 of the 5 patients removed alcohol in substantial amounts. In the fifth patient the liver for some reason did not. Small amounts of acetaldehyde were found in both peripheral and hepatic blood. The glucose concentrations remained relatively constant throughout, the hepatic vein concentrations being somewhat higher than those in the peripheral vein, as would

be expected. Blood lactate and pyruvate concentrations were difficult to interpret except for a consistently lower pyruvate concentration in the hepatic vein blood than in peripheral blood.

In the second study hepatic blood flow was measured in 17 "normal" hospital patients and 12 alcoholics with fatty livers but without "clinical signs of advanced cirrhosis." All were known to have consumed a poor diet, but none was jaundiced or febrile nor was ascites or abdominal collateral circulation evident in any. The study in the alcoholics was repeated after treatment in the hospital with a nutritious diet in addition to alcohol withdrawal and bed rest and, when clinical and laboratory evidences of liver disease had disappeared, over periods ranging from twenty-one to 116 days. In addition to hepatic blood flow measured by the same method as in the previous study, splanchnic oxygen consumption and cardiac output by the direct Fick method were also determined. A liver biopsy to estimate the degree of fatty infiltration (marked in 6 of the patients, moderate in 4) and hepatic fibrosis, which was not prominent in any, was done shortly after admission and close to the time of measurement of hepatic blood flow.

The results showed little if any effect of the fatty livers upon hepatic blood flow. Thus, although hepatic blood flow was slightly lower in the patients than in the control subjects, this was not statistically significant. Increases in the hepatic blood flow after treatment were observed in 9 subjects but were usually of small degree. An increase in the oxygen consumption by the liver (and splanchnic bed) occurred together with a decreased hepatic venous oxygen saturation, suggesting a centrolobular hypoxia and supporting Himsworth's hypothesis. It would have been more instructive had the dietary details of the treatment of these patients been included in the paper. It might also be commented that the patients studied, although showing pathologic fatty infiltration of the liver, did not have severe active liver disease by clinical examination. It may be that the questionably significant decreases in hepatic blood flow found in the patients after therapy would have been greater and of significant degree had patients with more clinical evidence of hepatic disease (but again without fibrosis) been studied.

These two studies indicate that, in the first place, alcohol increases the hepatic blood flow, which the authors felt was due not to increased cardiac output but to decreased peripheral resistance. Further, alcoholics who had consumed a poor diet and had fatty infiltration of the liver did not show a significant decrease in hepatic blood flow but did have a lowered hepatic venous oxygen saturation, which presumably compromised the nutrition of the centrolobular liver cells.

METABOLIC CHANGES PRODUCED BY DEXTRAN

The polysaccharide dextran has been utilized experimentally and clinically as a substitute for blood plasma in treatment of shock and diminished blood volume. The need for such a material is greatest during wartime when shock induced by battle wounds or injury becomes a common medical problem and when, also, it may be difficult, if not impossible, to provide and assure an adequate supply of human plasma. Furthermore, the frequent induction of serum hepatitis by the use of human plasma has made it desirable to seek substitutes for this important material in the treatment of shock. Intravenously administered dextran has a prompt effect in increasing blood volume and, therefore, has been referred to as a "plasma volume expander" (*Nutrition Reviews* **11,** *281 (1953)*). This property derives from its relatively high molecular

weight, which increases osmotic pressure and tends to retain water within the vascular system. In this sense only is dextran a substitute for plasma. It should be remembered that solutions of this material do not afford the electrolytes present in plasma.

Dextran is obtained from the capsular material of the bacterium *Leuconostoc mesenteroides*, and as isolated is material of mixed molecular weight. Partial hydrolysis has been utilized to afford preparations of smaller and less variable molecular weight for clinical use. The lack of homogeneity and frequent production of reactions have complicated the clinical usefulness of dextran and the evaluation of its metabolic effects.

A recently published paper is concerned with the metabolic effects of large quantities of dextran administered over a relatively long period of time (J. R. Jaenike and C. Waterhouse, *Circulation* **11,** *1* (*1955*)). These studies were conducted utilizing human subjects, who were free from evidence of metabolic or cardiovascular disease. Each person was in the hospital during the period of study. All were placed and maintained on a carefully controlled diet. The diet was planned to be low in calories and in content of carbohydrate, producing ketosis and negative nitrogen balance. Aliquots of foods were analyzed at intervals to provide quantitative data on the nutrients consumed. Similarly, the quantities of certain elements excreted in urine and feces were determined daily. By these means balance studies were carried out. Included were nitrogen, phosphorus, calcium, sodium, chloride and potassium balances. After suitable control periods and the establishment of metabolic equilibriums, dextran solutions were administered. Control periods varied from ten to eighteen days in length. The dextran was given as a 6 per cent solution in 0.9 per cent sodium chloride or as a 12 per cent solution in 0.5 per cent sodium chloride. Quantities of from 60 to 120 g. of dextran were administered daily for periods as long as ten days.

Since the persons who participated in these experiments were selected because they were apparently without physiologic abnormalities, it is interesting to note the clinical observations made on the group during the period of dextran administration. All gained weight, the gain in weight varying between 3 and 5 kg. in those who received as much as 720 g. of dextran during a six-day period. The increase in plasma volume produced headache in several subjects. Another event of more serious implication was the occurrence of a bleeding tendency in all subjects who received injections of 12 per cent dextran. This was associated with prolongation of bleeding time. Clotting time and prothrombin concentration were not affected. Still another event of grave import occurred in one person, a woman 58 years of age, who had a minor elevation of blood pressure but no previous evidence of heart disease. After the second day of dextran administration this subject developed manifestations of cardiac failure and it was necessary to discontinue her participation in the study.

Consistently, there was a pronounced decrease in the packed red blood cell volume and an increase in the calculated plasma volume in all of the experimental subjects. These changes were most striking in those persons who received solutions with greatest concentrations of dextran. The increase in plasma volume varied from about 30 to 90 per cent over control values. It is remarkable that plasma volume, expanded by dextran administration, remained so for prolonged periods after the discontinuance of dextran injection.

Serum protein concentration decreased from 22 to 53 per cent during the dextran period. There were greater reductions in circulating globulin than in albumin. In 4 subjects there was a compensatory rise in total circulating serum protein occurring two to three weeks after the administration of dextran was discontinued.

The quantity of dextran in blood plasma

and that excreted in the urine were determined. From 50 to 60 per cent was excreted and 11 to 32 per cent was retained and circulated in plasma. The remainder, or 16 to 34 per cent of the polysaccharide, could not be accounted for and was presumed to have been metabolized or to be present in extravascular fluid or body cells.

The effect of dextran on nitrogen and phosphorus balance was studied in seven experimental periods in 6 subjects. The low calorie diet given these persons produced negative balance for both nitrogen and phosphorus in five instances. During periods of dextran administration there was sparing of both nitrogen and phosphorus. The authors make the assumption that the sparing effect results from the metabolism of dextran. On this basis it is shown that, with the metabolism of one gram of dextran, from 60 to 80 mg. of nitrogen are spared. The effect of similar quantities of glucose on nitrogen balance was also studied to afford comparison. It was determined that about 16 mg. of nitrogen were spared per gram of glucose, a value only one fourth of that for dextran. The polysaccharide possesses a structure which would suggest ultimate utilization as glucose. Since it was observed that there was greater sparing effect of dextran on phosphorus than on nitrogen, it was thought likely that glycogen was being actively deposited. The chief obstacle to accepting this concept is the fact that the nitrogen-sparing effect of dextran greatly exceeded that of glucose. However, the authors are not too disturbed by this discrepancy and state that the observed findings might be accounted for in terms of comparative rates of metabolism of glucose and dextran. In these subjects with caloric deficits glucose would be utilized readily as an energy source, whereas dextran would be degraded more slowly but probably at a constant rate.

During control periods on the low calorie diets all persons developed ketosis with acetonuria, which lessened during dextran administration. However, blood ketones which were elevated during control periods were not significantly reduced by dextran. Glucose given to one subject had a much greater antiketogenic effect, again indicating more direct and rapid utilization of glucose than dextran.

In evaluation of electrolyte changes it was found that sodium and chloride were retained during the period that plasma volume expanded under the influence of dextran but were excreted in the period of diuresis which followed. Potassium was also retained, but there was no effect on calcium balance during the dextran period.

Other studies were concerned with kidney function. No evidence was found indicating compromise or impairment of renal blood flow or glomerular filtration. Moreover, no change in maximum tubular excretory capacity occurred consistently in these subjects during periods of administration of relatively large quantities of dextran.

The experiments described are of interest because they demonstrate that dextran produces metabolic effects distinct and apart from the physical effects resulting from macromolecular size. There are indications, also, that the polysaccharide itself is metabolized and does not persist as a totally inert material in tissues. A somewhat surprising finding is the persistence of increased plasma volume for relatively long periods after injections of dextran were discontinued.

FURTHER STUDIES ON COPPER IN WILSON'S DISEASE

That there are large and abnormal quantities of copper in the liver, brain and other tissues in Wilson's disease (hepatolenticular degeneration) is now well known

(*Nutrition Reviews* **7,** *255* (*1949*); **12,** *198, 304* (*1954*)). The finding of a decreased blood copper content along with a reduction in the copper-containing protein ceruloplasmin and a positive copper balance has led some workers to the hypothesis that there are an increased absorption of copper, diminished carrying capacity of the element in the blood, and deposition of the copper in the affected tissues. These lead to liver damage (cirrhosis), brain damage producing the neurologic findings, and renal disease causing the aminoaciduria which frequently occurs in this disease (*Ibid.* **7,** *115* (*1949*); **8,** *252* (*1950*)). Other workers have suggested that the loss of amino acids might be nutritionally limiting and bring about the cirrhosis and perhaps the brain damage as well.

Study of the two fractions of copper in the plasma, one tightly bound to protein, ceruloplasmin, and the other relatively free, the so-called "direct-reacting fraction" (*Nutrition Reviews* **11,** *296* (*1953*)) in Wilson's disease led to the discovery that, although the ceruloplasmin and therefore the ceruloplasmin-bound copper are regularly low in concentration, there is an increase in the "direct-reacting fraction." These and other findings have been presented by G. E. Cartwright *et al.* (*J. Clin. Invest.* **33,** *1487* (*1954*)).

In line with the finding of an increase in the direct-reacting serum copper is the observation that spinal fluid copper in 6 cases ranged from 10 to 24 micrograms per 100 ml. with a mean of 18, the normal range being 3 to 9 with a mean of 6 micrograms per 100 ml. As might be expected, all of the copper reacted directly with the reagent used for its determination (sodium diethyl-dithiocarbamate). The copper content of the red blood cells was essentially normal, but the tissues of a patient who died during the study showed marked increase in copper content of all tissues except heart muscle, being particularly high in liver and brain. As had been shown by W. T. Zimdahl, I. Hyman, and E. D. Cook (*Neurology* **3,** *569* (*1953*)), a positive copper balance was found in 4 subjects with hepatolenticular degeneration studied, compared with 3 normal controls and 2 patients with rheumatoid arthritis. A slight positive copper balance was noted in rheumatoid arthritis, but it was less than half that occurring in Wilson's disease.

As has been noted often, an increased excretion of copper in patients with Wilson's disease was observed compared to normal individuals. In an effort to increase further the urinary excretion of copper four agents were administered: British Anti-Lewisite (BAL) intramuscularly; casein hydrolysate intravenously; calcium versenate by mouth, or intravenously; and potassium sulfide by mouth. The latter agent produced little or no increased copper excretion. Calcium versenate by mouth produced some increase in one patient but none in another, and irregular although not marked increases of copper excretion were observed after intravenous administration of calcium versenate. BAL and casein hydrolysate both gave an excellent copper diuresis which was enhanced when these two substances were given together.

In this connection, J. P. Mahoney *et al.* (*Proc. Soc. Exp. Biol. Med.* **88,** *427* (*1955*)) have confirmed the hypothesis of C. Bishop, W. T. Zimdahl, and J. H. Talbott (*Ibid.* **86,** *440* (*1954*)) that there is a renal defect relative to the reabsorption of urate in patients with Wilson's disease leading to a lowered serum concentration of uric acid. They found an increased amount of uric acid in the urine which was heightened after the intravenous injection of uric acid. In this study there seemed to be no relation between the renal excretion of copper and that of uric acid, suggesting that uric acid does not bind the copper in this situation.

From these data and others in the literature the authors conclude that the primary defect in Wilson's disease is a

deficiency in the formation of the plasma copper-containing protein, ceruloplasmin, leading to increased copper absorption from the gastrointestinal tract, accumulation of copper in the brain, liver and cornea, and thus to the lesions in these areas characteristic of the disease. They believe that the renal defect in reabsorption of amino acids and peptides is due to an accumulation of copper in the kidney, and increased urinary copper excretion is due to chelation (combining) with amino acids and peptides. Enhanced excretion of copper by the administration of protein hydrolysate intravenously would seem to be a further step in rational treatment.

PHOSPHORUS REQUIREMENTS OF THE CHICKEN

The importance of phosphorus in the growth and reproduction of animals is well known (*Nutrition Reviews* **4,** *195* (*1946*); **6,** *315* (*1948*)). However, much work remains to be done on the determination of the exact phosphorus requirements of most animals as well as man. Many factors are involved and it is doubtful if well defined values for requirements can be given without an equally well defined set of conditions by which the requirements were determined.

Studies on the phosphorus requirement of the chicken have pointed out the relative unavailability of phytin phosphorus (*Nutrition Reviews* **8,** *317* (*1950*)). Also, it is known that the dietary level of vitamin D and calcium as well as certain other minerals may affect the phosphorus requirements of poultry (*see* review by H. J. Almquist, *Poultry Sci.* **33,** *936* (*1954*)). The Committee on Animal Nutrition of the National Research Council (H. R. Bird *et al.*, "Nutrient Requirements for Poultry," *National Academy of Sciences—National Research Council, Publication 301* (*1954*)) suggests a requirement in the ration of 0.6 per cent of phosphorus for young chickens and for laying and breeding hens. Up to about half this amount of phosphorus may come from organic sources. This suggested requirement has been studied recently using two different types of diets.

W. F. O'Rourke, P. H. Phillips, and W. W. Cravens (*Poultry Sci.* **34,** *47* (*1955*)) have studied the phosphorus requirements of chickens and pullets at different stages of growth. Practical diets were used with ample amounts of calcium and vitamin D provided. The effect of various levels of phosphorus on growth, inorganic blood phosphorus, bone ash and egg production was used in evaluating the phosphorus requirement.

It was found that the phosphorus requirement of growing chickens decreased with age, as would be expected. Cockerel chicks up to eight weeks of age had a dietary requirement of 0.75 per cent of phosphorus, higher than the value set by the National Research Council (*loc. cit.*). The requirement for pullet chicks up to this age was about 0.6 per cent. After eight weeks only 0.5 per cent or less of phosphorus (possibly as low as 0.35 to 0.43 per cent in the later stages of growth) in the ration was required. This is less than the National Research Council's figure of 0.6 per cent for growing birds.

Studies were made with laying pullets fed a practical basal ration containing 0.35 per cent of phosphorus and 15.5 per cent of protein. Calcium, as oyster shell, was fed by free choice (the usual method of feeding calcium to hens). The results showed that the stress of egg production did not appear to increase the phosphorus requirement over 0.35 per cent, the amount present in the basal ration. Also, body weight, average age at first egg, hatchability and egg weight were not affected by the various levels of phosphorus tested. The actual requirement of

pullets could not be determined in this study since the amount necessary was less than the level of phosphorus in the basal diet.

Although feed consumption values were not given, it is known that hens consume roughly 150 g. of feed per day. Thus, the amount of phosphorus present in the basal ration (over 0.5 g. of phosphorus per day) is considerably greater than the daily phosphorus output in each egg (about 0.11 g.).

In other studies from the same laboratory, laying hens were fed a purified diet instead of a natural diet (W. F. O'Rourke, H. R. Bird, P. H. Phillips, and W. W. Cravens, *Poultry Sci.* **33,** *1117 (1954)*). The basal diet contained only 0.19 per cent of phosphorus, over half of which was present as phytin phosphorus. With this diet a reduction in egg production and hatchability of eggs was clearly evident in just a few weeks. These deficiency signs were overcome or prevented and normal results were obtained by the addition of inorganic phosphorus to the ration to make a total of 0.30 per cent of phosphorus (approximately 0.18 per cent nonphytin and 0.12 per cent phytin phosphorus).

Eggs obtained from pullets fed the low and the high phosphorus diets were analyzed for phosphorus by the Fiske and SubbaRow method. Although the authors present no data, they state that no differences were found in the phosphorus content (presumably total phosphorus) of eggs from the two groups. This suggests that either the proportion of the various phosphorus-containing compounds were adversely affected in the deficient eggs or that levels of other compounds, not containing phosphorus but which normally require phosphorus for their synthesis, were reduced. The authors state that the injection of "phosphorus-containing compounds" into the deficient eggs gave no improvement in hatchability, but no information is given as to the types of compounds used.

Several other weaknesses can be pointed out in these otherwise sound investigations. Although values are given for the level of phosphorus in the various basal rations the authors do not indicate how this value was determined. In the absence of such information one must presume that the levels were calculated from standard tables. It would seem important actually to make a phosphorus determination of the basal rations as long as rather exact requirements are being determined. Another point in this work which is questionable and which will require additional evidence is the level of phosphorus required up to eight weeks of age. The data presented to show that cockerel chicks have a dietary phosphorus requirement of 0.75 per cent rather than 0.60 per cent for maximum growth rates are not very convincing in the absence of statistical treatment of results. Bone ash was not improved with ration levels of phosphorus above 0.60 per cent, according to the authors.

These studies on the phosphorus requirement of chickens are significant because they demonstrate the importance of this mineral for hatchability of eggs. Also, the results show that the accepted value for phosphorus requirements of hens (0.60 per cent of the ration) appears to be considerably higher than necessary. As the authors indicate, the need for further investigations on the requirements for this important mineral is emphasized by this work.

CHOLINE DEFICIENCY AND MUSCULAR DYSTROPHY IN THE RABBIT

There have been few studies on choline deficiency in the rabbit, in contrast to other species, until the reports of E. L. Hove, D. H. Copeland, and W. D. Salmon (*J.*

Nutrition **53,** *377 (1954)*) and of Hove and Copeland (*Ibid.* **53,** *391 (1954)*) on acute and chronic choline deficiency. In addition to yielding information on the choline requirement of the rabbit, these studies showed effects of choline deficiency on the vitamin E requirement of the rabbit, and the occurrence of muscular dystrophy in chronic choline deficiency which are of interest.

Acute choline deficiency was produced on a diet which contained 30 per cent methanol-extracted peanut meal, 6 per cent purified casein, 4 per cent salt mixture, 29.9 per cent sucrose, 0.1 per cent cystine, 10 per cent roughage (processed rice bran), 19 per cent lard, and 1 per cent cod liver oil. To this were added the necessary vitamins except choline, folacin and vitamin B_{12}.

This diet contained only 0.006 per cent choline. When fed to weanling rabbits, growth soon ceased. Outward appearance of the animals remained good except for hair loss, which was complete in one instance. There was no weight loss before the animals died. A level of 0.12 per cent choline in the diet supported excellent growth. DL-methionine at a level of 0.4 per cent stimulated growth, but not markedly. Addition of folacin and vitamin B_{12} as well as methionine enhanced growth still further, but not so much as a level of 0.04 per cent choline. Monomethylaminoethanol, but not ethanolamine, when fed with methionine gave an excellent response. Adult animals placed on the choline-deficient diet lost weight promptly and severely until death occurred in approximately sixty days.

In all the animals which died of choline deficiency, the livers were cirrhotic and had fat contents as high as 58 per cent. Observed grossly, the livers were pale in color and had the characteristic hobnail surface. Kidney hemorrhage was absent but tubular necrosis gave evidence of renal involvement.

The blood plasma was described as having a bright golden yellow color, yet the van den Bergh blood test for bile pigments was negative and there was no jaundice. The Ehrlich reaction in the urine was strongly positive, but ether solubility studies and paper chromatography indicated that the substance responsible was porphobilinogen rather than urobilinogen. A mild anemia, partially prevented by methionine, but not affected by folacin and vitamin B_{12} addition to the diet, was evident in the choline-deficient rabbits.

The authors concluded that a level of 0.13 per cent choline in the diet represented the requirement of the rabbit. More than this did not affect the growth rate while 0.05 per cent was clearly suboptimal.

In their second paper Hove and Copeland (*loc. cit.*) described the occurrence of muscular dystrophy in choline-deficient rabbits. When the weanling rabbits at 4 weeks of age were placed on the diet, the dystrophy developed after seventy to one hundred days. The animals showed typical muscular weakness, particularly in the hind limbs, decreased urinary excretion of creatinine, and an increased output of creatine. The creatine output, while fluctuating markedly, showed a steady increase during the deficiency period. Muscle tissue taken from dystrophic rabbits and examined histologically showed swelling of the muscle fibers and loss of the characteristic striations. This was followed by diffuse hyaline degeneration and an increase in fibrous connective tissue. The picture is clearly similar to that seen in the dystrophy of vitamin E deficiency, yet the administration of choline caused the muscular weakness to disappear in four days and the creatine excretion dropped promptly to normal levels.

It was clear to the investigators that the muscle dystrophy observed might have been due to a secondarily induced vitamin E deficiency, particularly because of the accompanying liver cirrhosis. Indeed they demonstrated the low plasma tocopherol levels which occur both in rats and rabbits with liver cirrhosis due to choline deficiency. It was also shown that the vitamin E

requirement of the choline-deficient rabbits was considerably increased. Nevertheless, choline-deficient animals developed muscle dystrophy even when receiving 10 mg. of vitamin E per day, and the blood and tissue tocopherol levels were adequate under these circumstances.

Thus the investigators believe that choline deficiency per se produces a muscular dystrophy in rabbits. While they consider that the deficiency might be an indirect effect of the liver cirrhosis because of less functional liver tissue, the rapid cure of the dystrophy upon choline administration would seem too much to expect if it depended upon reversal of liver damage. The authors have therefore indulged in some speculation that the primary cause of muscle dystrophy is lack of acetylcholine, known to be vital in the transmission of the nerve impulse, and on this ground a deficiency of choline would be expected to produce muscle dystrophy. In order to make a unified concept in which both choline and vitamin E deficiency might lead to muscle dystrophy, the authors have suggested that the tissue peroxides in vitamin E deficiency could cause oxidation and inactivation of coenzyme A which is essential in the formation of acetylcholine. On the same grounds a deficiency of pantothenic acid might be expected to lead to muscle dystrophy. Unfortunately neither of these suggestions is well founded in fact at present.

Nevertheless it is true that efforts to benefit progressive muscular dystrophy in man by administration of vitamin E have not been successful to date. While it is even more unlikely that dietary deficiency of choline is responsible for human muscle dystrophy, the relationship among choline, liver cirrhosis, and muscle dystrophy demonstrated by Hove and Copeland may open new avenues of approach to an understanding and improved therapy of the muscle disorder.

FOLIN'S CONCEPT OF PROTEIN METABOLISM

The early observations of R. Schoenheimer and his colleagues upon the dynamic state of the body proteins (Schoenheimer, "The Dynamic State of Body Constituents," *Harvard University Press, Cambridge (1942)*) led them and others to deny the possibility of a distinction between the endogenous and exogenous protein metabolism as postulated by O. Folin (*Am. J. Physiol.* **13,** *117 (1905)*). H. H. Mitchell (*see Nutrition Reviews* **2,** *169 (1944)*) has previously indicated his belief that the observations on the dynamic state of protein metabolism are not necessarily out of harmony with Folin's original concept. In a recent paper he has reviewed some evidence and presented additional data to support the concept of a real dichotomy in protein metabolism (*J. Nutrition* **55,** *193 (1955)*).

The data presented by Mitchell include urinary nitrogen excretion figures for rats and pigs, the pig data taken from the paper of A. Columbus (*Arch. Tierernährung* **1,** *84 (1950)*), at varying levels of intake of several proteins. When the excretion per unit surface area is plotted against absorbed nitrogen per unit surface area a straight line relationship is observed within the range from zero to levels which exceed the capacity of the animal to store protein. The range over which the linear relationship holds depends upon the biologic value of the protein. In the rat data presented, for example, the linear regression holds up to 55 mg. of nitrogen per 100 square centimeters when egg protein was fed but up to 85 mg. of nitrogen per 100 square centimeters when beef protein was used. Similarly, the data upon rats and pigs confirm the findings of J. B. Allison and J. A. Anderson (*J. Nutrition* **29,** *413 (1945)*), and of Allison, R. D. Seeley, J. H. Brown, and Anderson (*Ibid.* **31,** *237 (1946)*) that nitrogen balance

bears a linear relationship to nitrogen intake over a similar range of intakes. The slope of this line, as Allison and his group have emphasized, is a measure of the biologic value of the protein fed.

The point that Mitchell stresses, of course, is that these straight line regressions, which transect the Y axis at the same point obtained when a nitrogen-free diet is fed, are not intelligible unless the endogenous nitrogen excretion does in fact remain constant. The data presented clearly support this concept. The most serious challenge to the constancy of endogenous nitrogen excretion comes from studies such as those of M. Brush, W. Willman, and P. P. Swanson (*J. Nutrition* **33,** *389 (1947)*) and Allison and co-workers (*loc. cit.*) and others who have demonstrated actual reductions in the urinary nitrogen excretion below endogenous levels by the incorporation of certain high quality proteins or methionine alone in the diet. Mitchell believes that these results might be explained by an accelerated endogenous nitrogen metabolism caused by liver dysfunction associated with the feeding of a protein-free, high fat diet in the studies mentioned above, and a beneficial effect of methionine on the liver function. Indeed the urinary nitrogen figure obtained by Allison *et al.* on the nitrogen-free diet of about 2.6 g. per square meter per day is unusually high. A value of 1.5 g. per square meter per day, which approximates the value they obtained when methionine or egg protein was fed, is more in line with prior estimates of endogenous nitrogen of several authors, notably D. B. Smuts (*Ibid.* **9,** *403 (1935)*).

Whether the values obtained by Brush *et al.* are also high is not readily apparent from the data they present. The effect of methionine upon endogenous nitrogen has not been found in human beings (R. M. Johnson, H. J. Deuel, M. G. Morehouse, and J. W. Mehl, *J. Nutrition* **33,** *371 (1947)*), and the endogenous nitrogen excretions obtained by Johnson *et al.* and by M. Bricker, H. H. Mitchell, and G. M. Kinsman (*Ibid.* **30,** *269 (1945)*) approach the levels found by Smuts (*loc. cit.*) for several different species.

Mitchell also cites the isotope studies of H. Tarver and L. M. Morse (*J. Biol. Chem.* **173,** *53 (1948)*) with radioactive sulfur, of R. C. Thompson (*Ibid.* **197,** *81 (1952)*; **200,** *731 (1953)*) with tritium, and of H. D. Hoberman (*Ibid.* **188,** *797 (1951)*) with heavy nitrogen as examples of recent demonstrations of the dichotomy of body metabolism. Hoberman, for example, concluded "that in fasting animals two reservoirs of protein contribute nitrogen to the amino acid pool. One reservoir interacts rapidly with the amino acids of the tissues, while another portion of the body proteins would appear to be susceptible to virtually irreversible hydrolysis." Thus recent work with isotopes has clarified the nature of endogenous metabolism, and as C. P. Berg ("Proteins and Amino Acids in Nutrition," M. Sahyun, editor, *p. 290, see esp. p. 297, Reinhold Publishing Corp., New York (1948)*) has said, "The change needed is one of point of view." The concept itself is apparently valid, as valid as a concept can be that is not susceptible to direct experimental approach.

Finally, Mitchell has emphasized that it is the concept of an endogenous metabolism of nitrogen which is directly related to basal metabolism, and which is replaceable by dietary protein, which makes sense out of comparative data on protein requirements. Studies in experimental animals become particularly valuable when they can be related to a common denominator that is also applicable in human nutrition. The minimum protein requirement is probably as well defined as the minimum requirement for any nutrient. However, as would be expected, the minimum level of excretion is reached only after a certain degree of body depletion. The major problem in protein nutrition is to define the benefits of intakes above minimum and how much of a margin above minimum is necessary for optimum health.

NIACIN AND AMINO ACID IMBALANCE

The deleterious effect of amino acid imbalance produced by the addition of certain amino acids or proteins to diets limiting in protein or niacin has been observed by many workers. C. A. Elvehjem and W. A. Krehl ("American Medical Association Handbook of Nutrition," *Chapt. 18, Blakiston Co., New York (1951)*) point out that growth inhibition in rats was obtained by the addition of tryptophan-free proteins or acid hydrolysates to a diet containing 9 per cent casein and no niacin. Glycine supplements also inhibited growth. L. V. Hankes, L. M. Henderson, and Elvehjem (*J. Biol. Chem.* **180,** *1027 (1949)*) observed growth suppression with a niacin-free diet containing 9 per cent casein upon the addition of threonine or phenylalanine, and suggested that the effect might be explained by assuming that when these essential amino acids were added to the diet the low tryptophan level already present might become limiting for protein synthesis and that little would be available for niacin synthesis. Thus a niacin deficiency would be induced. O. J. Koeppe and Henderson (*J. Nutrition* **55,** *23 (1955)*) have investigated this possibility in more detail.

Most of the studies were done with a protein-free diet containing a mixture of amino acids. The so-called "complete diet" provided all essential amino acids at levels equal to or greater than those required by the rat (W. C. Rose and M. Womack, *J. Biol. Chem.* **166,** *103 (1946)*), with the exception of tryptophan which was provided at either 0.10 or 0.11 per cent of DL-tryptophan. Various nonessential amino acids were added to provide a total amino acid content of the diet of approximately 18 per cent. The particular amino acid studied was varied as desired. The remainder of the diet was composed of sucrose, 5 per cent corn oil, 4 per cent salt mixture, and a niacin-free vitamin mixture. The growth of young rats weighing 45 g. at the start of the experiment was followed for a four-week period.

The levels of threonine, lysine, isoleucine, leucine and phenylalanine were varied in different experiments. In the studies with threonine, lysine and leucine the results were rather clear-cut and similar. At the lower levels fed, which varied with the amino acid under study, growth was poor. As the level of the amino acid was increased, growth improved up to a certain point, and then at higher levels poor growth was again obtained. For example, with 0.5 per cent threonine the average gain was 15 g. in four weeks; at 0.6 per cent, 30 g.; at 0.7 per cent, 21 g.; and at 0.8 per cent, only 10.5 g. When niacin was added to the latter diet containing 0.8 per cent threonine, the animals gained 63 g. in the same period. Thus it appeared that an optimum level was reached for each of these amino acids and at higher levels a niacin deficiency was induced.

The studies with isoleucine were less satisfying. Changing the isoleucine level had no significant effect upon the growth rate, although additions of niacin indicated that the animals were suffering from a deficiency of this vitamin. The authors then tried the same type of experiment but with levels of tryptophan at 0.105 and 0.11 per cent rather than the 0.10 per cent level used in the other experiments. At the highest level of tryptophan, growth was suppressed somewhat as the level of isoleucine was increased, and again good responses were obtained with niacin additions. Similarly, in studies with phenylalanine, growth was so poor that growth suppression at higher levels was not demonstrated. The authors feel that perhaps the level of tyrosine in the diet might be of significance in these studies. The much poorer growth obtained in the work with isoleucine and phenylalanine is not explained.

A similar study was done using a diet containing 10 per cent gelatin supplemented with various missing amino acids. Growth inhibition with increasing levels of threonine

which was counteracted by niacin supplements was demonstrated.

As indicated above, the authors suggest that the results may be explainable if with increasing levels of a certain amino acid the tryptophan in the diet is drawn toward protein synthesis and thus becomes unavailable for niacin formation. Examination of the growth curves showed that most of the growth occurred during the first week and that practically no growth or even loss of weight occurred during the subsequent weeks. This puts a severe strain upon the theory, since one might assume that when growth ceased more tryptophan would become available for niacin synthesis. It is not quite clear what the authors mean to imply by the statement that "It is reasonable to assume that pyridine nucleotides are involved in the reactions by which tryptophan gives rise to niacin. This may account for the failure of the animal to resume growth when tryptophan becomes available for niacin synthesis as a result of reduced protein synthesis." The authors also suggest that loss of appetite, as a result of the niacin deficiency, might contribute to the failure of animals to resume growth when protein synthesis ceases. Paired feeding experiments were run and as might be expected the differences obtained were considerably less than with ad libitum feeding. The growth of the threonine-supplemented group was less than that of those without threonine in each of four experiments where food intake was equalized, but whether the growth rates are significantly different is not clear. Whether the results support the hypothesis of the authors or not is uncertain, since one must expect groups which have the same food intake to be more nearly alike than when this restriction is not practiced, and greater statistical control is required.

The explanation of an induced niacin deficiency by an increase in protein synthesis in the face of a limited tryptophan supply is weakened by the failure of the isoleucine and phenylalanine experiments to conform to the theory. Also, it is doubtful that the previously observed effect of glycine supplements causing the same induced niacin deficiency can be explained upon this basis. Nevertheless the studies are of considerable interest. They emphasize the complexities of the growth process and the interrelations among nutrients. Hypotheses which breed new work are necessary and worthwhile as long as they are recognized as hypotheses and do not become accepted as adequate explanations.

THE VITAMIN B_{12} GROUP

Cyanocobalamin (vitamin B_{12}) consists of a relatively high molecular weight cobalt complex (C_{46-49} H_{67-75} O_{6-7} N_{11}) and a nucleotide the nitrogenous base of which is 5,6-dimethylbenziminazole. From a variety of materials of microbiologic origin an extensive series of related compounds has been isolated. They appear to differ from cyanocobalamin largely in the nature of the nitrogenous base of the nucleotide portion. In a number of cases the nature of the nitrogenous base has been elucidated. Recent studies have also shed some light on the method of synthesis of the nucleotide portion by microorganisms.

K. H. Fantes and C. H. O'Callaghan (*Biochem. J.* **59,** *79 (1955)*) have reinvestigated the uptake of o-phenylenediamine by *Streptomyces griseus* from a synthetic medium. Assay of the medium after four days of incubation (using a strain of *Escherichia coli* as well as *Lactobacillus leichmannii* as assay organisms) showed that the phenylenediamine had doubled the apparent vitamin B_{12} activity. High concentrations of the diamine were, however, inhibitory. In additional experiments the true vitamin B_{12} content of the medium was determined after chromatographic separation and was found to be diminished by incuba-

tion with o-phenylenediamine. This indicated strongly the formation of a new growth factor differing structurally from vitamin B_{12} but possessing qualitatively the same microbiologic activity for *E. coli* and *L. leichmannii*. Formation of the new factor could be prevented by adding to the medium 1,2-diamino-4,5-dimethylbenzene, presumably a precursor of vitamin B_{12} itself.

The new factor was isolated by elution from paper chromatograms and crystallization from aqueous acetone. Its microbiologic potency for *E. coli* and *L. leichmannii* was about twice that of vitamin B_{12}. Preliminary work on its structure suggested that it differed from vitamin B_{12} in the absence of the two methyl groups of the benziminazole moiety. A footnote calls attention to the fact that the new substance was fully active in 3 pernicious anemia patients.

Another approach to the study of the biosynthesis of vitamin B_{12} and related compounds was reported by J. E. Ford, E. S. Holdsworth, and S. K. Kon (*Biochem. J.* **59,** *87 (1955)*). These workers made use of a strain of *E. coli* (113–3) which requires vitamin B_{12} for growth. This organism is capable of synthesizing vitamin B_{12} (cyanocobalamin) if supplied with factor B (the non-nucleotide portion of the molecule) and 5,6-dimethylbenziminazole. To the latter it also attaches ribose and phosphate. As is shown in this study, the organism will attach to factor B a variety of nitrogenous bases other than 5,6-dimethylbenziminazole to form vitamin B_{12}-like compounds. The various compounds formed were isolated from the bacterial cells and then separated and tentatively identified by paper chromatography. The test organism was found capable of synthesizing factor A by coupling factor B (the non-nucleotide portion of vitamin B_{12}) with 2-methyladenine. Pseudovitamin B_{12} was produced from factor B and adenine. Factor B alone gave rise to factor C. Factors A, B and C and pseudovitamin B_{12} are often found together with cyanocobalamin where bacterial fermentation occurs (rumen, gut, feces). Each of these substances contains the same cobalt-peptide complex (factor B) linked in the case of factor A with a nucleotide of 2-methyladenine, in the case of pseudovitamin B_{12} with a nucleotide of adenine, and in the case of vitamin B_{12} itself with a nucleotide of 5,6-dimethylbenziminazole. A number of new vitamin B_{12}-like compounds were synthesized by *E. coli* by substituting for dimethylbenziminazole such nitrogenous bases as 5-methylbenziminazole, benziminazole, benzthiazole, riboflavin and others. The factor obtained with benziminazole is perhaps identical with that obtained by Fantes and O'Callaghan (*loc. cit.*) with the use of o-phenylenediamine. Several of the compounds formed were found to support the growth of *Ochromonas malhamensis*, an organism thought hitherto to respond only to vitamin B_{12} (cyanocobalamin) itself.

F. B. Brown *et al.* (*Biochem. J.* **59,** *82 (1955)*) have applied extensive chromatographic methods to the cobalt-containing compounds of pig and calf manure. They demonstrated the natural occurrence of factors A, B and C, and pseudovitamin B_{12} in addition to vitamin B_{12} itself. They also found an additional group of red cobalt complexes which they labeled factors D to I. Factor A was again demonstrated to contain 2-methyladenine in the nucleotide portion of the molecule, while pseudovitamin B_{12} was shown to contain adenine. Factor G was found to be identical with deaminated pseudovitamin B_{12} and thus presumably contains hypoxanthine as its nitrogenous base. Similarly, factor H was shown to be identical with deaminated factor A and presumably contains 2-methylhypoxanthine in its nucleotide portion. The basic non-nucleotide portion of these compounds appears to be identical (factor B). The authors carefully point out that a substance such as factor H need not necessarily be synthesized by the intestinal microorganisms but might arise by enzymatic deamination of factor A.

It will be of interest to see whether the numerous naturally occurring vitamin B_{12}-

like compounds each plays a significant metabolic role or whether a substantial number are only by-products formed during the synthesis of a few biologically active ones.

EFFECT OF HUNGER ON HYPOTHALAMIC METABOLISM

Use of classic technics of neurophysiology involving controlled destruction or stimulation of discrete areas in the brain has yielded much valuable information about the central mechanism concerned with the regulation of food intake. It has been shown that destruction of the paired ventromedial nuclei of the hypothalamus in certain experimental animals is followed by development of obesity (J. R. Brobeck, *Physiol. Rev.* **26,** *541 (1946)*). Such obesity is largely the result of an increased food intake. Destruction of the lateral hypothalamic nuclei is followed by cessation of feeding, while stimulation of these same lateral nuclei results in a marked increase in food intake (J. M. R. Delgado and B. K. Anand, *Am. J. Physiol.* **172,** *162 (1953)*).

Thus, the existence and approximate location of important neural centers having to do with energy balance have been determined by traditional physiologic methods. However, in order to obtain information about the mechanisms whereby such centers operate a more subtle approach is required. For example, direct metabolic studies of the neural areas concerned with energy balance might be expected to provide information about the way such centers detect the body's need for more food.

Previous studies on the biochemical aspects of food intake regulation (J. Mayer, *Physiol. Rev.* **33,** *472 (1953)*) have suggested that the "urge to eat" may be linked to the body's supply of carbohydrate ("glucostatic theory"). Since the central nervous system utilizes carbohydrate as virtually its sole source of energy, it has been proposed as a corollary of the glucostatic theory that the brain centers (glucoreceptors) which are postulated to respond to carbohydrate privation must be more representative of certain peripheral tissues in their permeability to glucose than of brain tissue. This follows because there is no evidence that in hunger states the brain, taken as a whole, is deficient in carbohydrate. Indeed, the economy of the body is adjusted so as to prevent cerebral carbohydrate deficiency from occurring under any circumstances.

S. Larsson and A. Forssberg (*Acta physiol. Scandinav.* **32,** *Suppl. 115, 1 (1954)*) have performed a number of interesting and unique experiments to test the hypothesis that the hypothalamic centers concerned with energy balance differ from brain tissue generally in their metabolic responses to fed and fasted states. The authors report their experiments in a monograph which is divided into two main parts. The first part describes extension of classic studies involving electrical stimulation of certain parts of the brain stem and administration of intrahypothalamic injections to induce an increase in food intake. The second part deals with biochemical experiments on rats, designed to determine whether any metabolic changes can be demonstrated in the hypothalamus which correlate with states of hunger or satiety. Specifically, these experiments involved (1) studies of isotope distribution, and (2) determination of chemical composition in the hypothalamic region of hungry and fed rats.

Radiophosphate (P^{32}) uptake was measured in the hypothalamic area of rats which contains the lateral and ventromedial nuclei. P^{32}-incorporation in this area was studied in fasted and fed rats and compared with phosphate incorporation in two control areas of the hypothalamus believed not to be concerned with food intake regulation. Hydrolyzable phosphate (creatine phosphate plus adenosinetriphosphate, ATP) concen-

trations also were measured in the same hypothalamic areas and preliminary studies on C^{14}-labeled glucose uptake in these areas were performed.

It was found that the hypothalamic area containing the ventromedial and lateral nuclei incorporated significantly more labeled phosphate in hungry than in fed rats. At the same time, the control areas showed less incorporation of radiophosphate during hunger than in the fed state. The ATP-phosphagen concentration was markedly higher in the "feeding center" area of hungry rats than in the control areas. An opposite situation was found in fed rats.

Several experiments involving injection of C^{14}-glucose also were done. Although these experiments were too few to be subjected to statistical treatment, the trend so far as C^{14} incorporation was concerned was similar to that observed in the P^{32} experiments. More activity was incorporated into the feeding center areas of the hungry rats than in the two control areas.

In summarizing their results, the authors emphasize primarily the fact that when P^{32} is used as a tracer the activity is incorporated into the feeding center area and the two control areas according to a characteristic pattern which distinguishes hungry rats from fed rats. They suggest that the increased ATP present in the feeding center areas is associated with a local increase of acetylcholine (Ach). The increased Ach would be expected to cause a local capillary dilatation which, in turn, would increase the amount of P^{32} available for uptake in the area. Thus, the increased P^{32} incorporation in the feeding center is believed to be due indirectly to an increase in local concentration of high-energy phosphate. The authors conclude their interpretation by recording their impression that the hunger state does not cause changes merely in the feeding center. Rather, the impression is gained of a "closely correlated chain of reactions in which various parts of the hypothalamic region participate." This entails a shift in ATP concentration from one part of the hypothalamus to another and consequently also a shift in the energy metabolism.

Although the demonstration of a metabolic pattern in the feeding center "area" which distinguishes hungry from fed rats is convincing, the interpretation the authors place on their findings is somewhat recondite considering the relatively small amount of information available on the subject to date. The practice of grouping the ventromedial and the lateral hypothalamic nuclei under the collective term "feeding center" may have some physiologic justification. However, the two sets of nuclei may well be as distinct in their biochemical behavior as their roles appear to be in regulating energy balance. Clearly, much more work must be done before the biochemical data obtained by Larsson and Forssberg can be integrated into an over-all theory of food intake regulation.

The finding of an increased content of high-energy phosphate in the feeding center area during hunger is consistent with the view that there is increased metabolic activity in some portion of this area as a consequence of "hunger." The nature of the stimulus which signals the need for more food remains obscure; however, it would appear the biochemical studies such as those initiated by Larsson and Forssberg offer the best approach to a solution to this problem.

THE NUTRITIVE VALUE OF COW'S MILK FOR THE RAT

Various studies have indicated that the nutritive value of cow's milk was higher during the season when the cows were on fresh pasture and declined as the length of

time in the barn increased (C. A. Elvehjem, E. B. Hart, H. C. Jackson, and K. G. Weckel, *J. Dairy Sci.* **17,** *763 (1934)*; F. E. Stirn, Elvehjem, and Hart, *Ibid.* **18,** *333 (1935)*; G. O. Kohler, Elvehjem, and Hart, *J. Nutrition* **14,** *131 (1937)*; **15,** *445 (1938)*). Other workers have also commented on the superior qualities of summer milk (T. P. Hilditch and H. Jasperson, *J. Soc. Chem. Ind.* **60,** *305 (1941)*; J. M. Lawrence, B. L. Herrington, and L. A. Maynard, *J. Nutrition* **32,** *73 (1946)*).

J. N. Bixby, A. J. Bosch, C. A. Elvehjem, and A. M. Swanson (*J. Agr. Food Chem.* **2,** *978 (1954)*) have examined a series of factors which might affect the nutritive value of cow's milk. In 18 experiments that extended over a two-year period, the primary objectives were to study the effect of season, pasteurization and further heat treatment upon the nutritive value of cow's milk and to determine the effect of various supplements to a cow's milk diet on the growth and general well-being of the albino rat. The various milk diets were mineralized with 3.0 mg. of iron, 0.15 mg. of copper and 0.15 mg. of manganese per 100 ml. of milk, using iron pyrophosphate, copper sulfate and manganous sulfate, respectively, and fed ad libitum twice daily. Most of the experiments were conducted for a six-week period, at the end of which time the animals were killed and the livers were removed for fat analysis by a wet ether extraction method. A control diet composed of grains and protein concentrates with supplements of calcium carbonate, sodium chloride and liver concentrate was used for comparative purposes. The average weekly gain in weight during the six-week test period for the raw milk, the pasteurized milk and the homogenized milk diets indicated that the growth stimulated by these milk diets was good, but was slightly less than that obtained on the stock diet. No difference in growth rate was observed from raw, pasteurized and homogenized milk which had been derived from the same source. No seasonal variation in the ability to promote growth was noticed in either raw, pasteurized, or homogenized milk.

The results in these growth experiments indicated that the rats on the milk diets at the end of the six-week test period had liver fat contents that were higher in all groups than in weanling rats or in those rats which had been fed for the same period of time on the stock ration. The average liver fat value for rats fed the stock ration was 4.3 per cent while that for all rats fed the various milk diets was 7.2 per cent. The liver fat values did not differ significantly between the raw and the pasteurized milk groups, but were slightly lower for rats fed homogenized milk. The data presented in regard to seasonal variation and effect on the liver fat accumulation were limited, but no significant differences were observed. It was believed that the lower liver fat values among the rats fed homogenized milk could be attributed to the fact that the homogenized milk did not separate to provide a thick cream layer which the rats could eat preferentially.

The present investigators drew attention to the discrepancy between their current results and the earlier data from the same laboratory. They suggest that the only apparent explanation for the difference may be that winter milk may be of higher nutritive value now than it was twenty years ago. They make the reasonable statement that the production of milk of a higher nutritive value today is not unlikely in view of the greater knowledge of plant and animal nutrition and the precautions taken to design and offer complete diets to dairy cows.

When the mineralized and pasteurized milk diet was supplemented with choline at two levels, by a combination of folacin, vitamin B_{12} and choline, by threonine, or by threonine, choline and methionine simultaneously, or by sucrose additions, no influence was noted on the average weekly gain in body weight. A magnesium supple-

ment at 48 mg. per 100 ml. of milk depressed growth slightly. Supplements of methionine and lysine caused slight increases in the average growth rate above that of the animals receiving only mineralized milk. The influences of these supplements upon liver fat percentage are somewhat indecisive since there was a wide variation in the fat content of the livers of the rats fed mineralized milk only. The only group with an appreciable reduction in per cent of liver fat was the one receiving folacin, vitamin B_{12} and choline, where a decrease from 6.9 to 5.1 per cent was observed. A similar but not quite so large decrease was noted in the group which received the mineralized milk diet into which additional solids, in the form of a partially delactosed skim milk powder, had been introduced. However, none of these reductions brought the liver fat percentage down to a level which was comparable to that of the rats which were receiving the control diet.

When the butterfat content of the milk diet was altered between the levels of 1 and 4.75 per cent, it was noted that the animals grew increasingly well up to a level of 4.0 per cent butterfat, but a reduced rate of growth was noted in the group which received the 4.75 per cent butterfat milk. The average percentage of liver fat in these groups increased from 4.9 in the group receiving the milk with the 1 per cent butterfat to a level of 9.8 per cent in the group with the highest butterfat level. When milk was heated for thirty minutes at either 145° F. or 165° F. after homogenization, no effect was noted upon the rate of growth. However, when the heat process was increased to 185° F. for thirty minutes, a 10 per cent reduction in growth was observed. None of these heat treatments caused any particularly striking influence upon the liver fat accumulation.

When either dried whole milk or dried whole milk which had been reconstituted was fed as the sole diet there was no deleterious influence upon the rate of growth. Likewise whole milk, sterilized at high temperature via the short-time process, gave equally good growth. The latter three procedures resulted in slightly lower percentages of liver fat.

When the influence of reproduction and lactation was studied among female rats fed raw and pasteurized milk for three generations, no difference in performance could be noticed between the animals which received the raw milk and the pasteurized milk. The weights of weanlings in the first litter of each generation were slightly lower than those in later litters. This is a common occurrence among young females in most breeding colonies. However, it is noteworthy that the average rate of growth among both male and female offspring in the second and third generations was appreciably lower than for the animals in the first generation. The reason for these lower rates of growth cannot be explained on the basis of the present findings. However, these data would seem to indicate that a depletion process was going on in the production of repeated generations. It would have been interesting to have known how weanling rats in the second and third generations would have performed if fed a stock ration, in comparison to their littermates which were maintained on the milk diet.

The authors draw attention to their view that the rat may not be the ideal experimental subject for testing the nutritive quality of milk. They noted that neither the cat nor the guinea pig accepts milk with any degree of willingness as its sole source of nutrients. In one experiment several young cats on a mineralized, pasteurized milk diet gained from 1000 to 1500 g. in an eight-month test period while others would not accept the milk and did not survive. They noted no difference in the performance of cats fed raw milk or pasteurized milk diets.

The difference in growth performance of weanling rats in the first generation in comparison with those of the second and

third generations is worthy of particular attention. In all probability, the members of the second and third generations had been partially depleted of some one or more nutrients. Obviously, the degree of depletion was not sufficient to prohibit reproduction and lactation. Oftentimes in nutrition research the potentialities of repeated reproductive cycles for the depletion of the experimental subjects in one or more nutrients is overlooked. Though the necessity to impose the reproductive stress as a means to deplete the members of future generations is laborious, it frequently is the only way to achieve the desired goal. It would be of real interest to attempt to define the causes of the reduced rate of growth among the offspring of the second and third generations in this experimental series.

ABSORPTION OF CHOLESTEROL BY RATS

The rat has been used extensively in determining the mode of absorption of a variety of compounds that have been implicated in the development of atherosclerosis. Cholesterol levels as high as those seen in rabbits developing atherosclerosis have been produced in the rat (*Nutrition Reviews* **11,** *152 (1953)*). However, the rats with these high cholesterol levels did not show true atheroma in the arteries.

L. Swell, D. F. Flick, H. Field, and C. R. Treadwell (*Am. J. Physiol.* **180,** *124 (1955)*) have studied the role of fat and fatty acids in the absorption of cholesterol and cholesterol esters. Adult rats that were raised on a chow diet were fed 25 per cent fat or fatty acids, 1 per cent sodium taurocholate and 2 per cent cholesterol. The latter were added in various combinations to the basal purified diet. When the basal diet containing soybean oils with iodine numbers ranging from 14 to 80 was fed alone, the blood cholesterol levels increased to the greatest extent in the rats receiving the oil with the highest iodine number. Even in the case of the latter rats, the increase was only from about 110 to 130 mg. per cent of total cholesterol. Most of this increase was in the ester fraction. For the diets containing oils with the lower iodine numbers, the change in blood cholesterol was so small that its significance is questioned.

When both 2 per cent cholesterol and 1 per cent sodium taurocholate were present in the diet, the addition of 25 per cent oleic acid increased the blood cholesterol level from about 110 to approximately 300 mg. per cent. Most of this increase occurred during the first seven days. The cholesterol level increased to almost the same extent when stearic acid was used as the source of fat. Linoleic acid and peanut oil were much less effective than oleic or stearic acid in increasing the blood cholesterol level. Linseed and corn oils were least effective in raising the blood cholesterol level. These findings indicate that the free fatty acids are much more effective than the neutral fats in facilitating the absorption of cholesterol by the rat.

This work indicates that the rat appears to handle free fatty acids without difficulty when they are incorporated as 25 per cent of the diet. This high level of free fatty acids, according to Swell and co-workers, had no apparent effect on the liver or other organs during the three-week experimental period.

In an effort to explain the role of the free fatty acids in facilitating the absorption of cholesterol, *in vitro* studies of the rate of enzymatic esterification of the cholesterol were carried out. Using a purified pancreatic cholesterol esterase, these workers found that the fastest rate of cholesterol esterification occurred in the presence of oleic and linoleic acid. Saturated acids such as stearic and palmitic when added to the enzyme

preparation resulted in much slower rates of esterification.

In other experiments (L. Swell, T. A. Boiter, H. Field, and C. R. Treadwell, *Am. J. Physiol.* **180,** *129 (1955)*) 1 per cent bile salt was added to the purified diet containing 25 per cent olive oil. When this diet was supplemented with 2 per cent cholesterol, the blood cholesterol level increased from approximately 110 mg. per cent to about 250. When the diet contained equivalent amounts of cholesterol esters as the butyrate, oleate, or linoleate esters, the blood cholesterol esters in the rats at the end of the three-week experiment were much lower than when the free cholesterol was fed. It is unfortunate that the experiments were terminated at the twenty-first day, since at that time the blood cholesterol levels in the rats receiving the cholesterol esters of oleic and linoleic acids were still increasing, while the levels in the other two groups had been stationary for the last week of the experiment. In the absence of bile salts, cholesterol levels in the blood increased to an extent that was only a small fraction of that seen in the preceding experiment.

On the basis of these studies, Swell and co-workers suggest that pancreatic cholesterol esterase, fat and bile are involved in the absorption of cholesterol. They believe that the bile functions both as a co-factor for cholesterol esterase and as a complexing agent for cholesterol and fatty acids. The fat provides the fatty acids which facilitate the absorption of cholesterol and stimulate the flow of bile. Pancreatic cholesterol esterase may be involved in esterifying or hydrolytic reactions either in the lumen, the wall of the intestine, or both. They suggest that the cholesterol must be in the free form before it can be absorbed and that the esters of cholesterol are split in the intestine. The latter hypothesis would confirm the work of J. H. Mueller (*J. Biol. Chem.* **27,** *463 (1916)*), who found that when cholesterol oleate was fed to dogs, most of the cholesterol in the intestine was in the free form. The cholesterol in the stomach, however, was present primarily in the ester form.

Unfortunately, these workers have not suggested how cholesterol is absorbed from the intestinal tract. Work of this type may eventually, however, provide us with a better understanding as to how dietary cholesterol is absorbed.

6-MERCAPTOPURINE AND NUCLEIC ACID SYNTHESIS

The importance which nucleic acid has assumed in modern biologic concepts makes the appearance of a potent antimetabolite affecting its synthesis a fact of considerable interest. Within the cell nucleus nucleic acid is associated with the chromosomes determining the hereditary characteristics of the cell. There is also evidence that certain nucleic acids are involved in the synthesis of proteins. Other functions of nucleic acids in regulating growth, development, and metabolic processes have been suggested. The nucleotide nature of a variety of coenzymes functioning in oxidative metabolism has long been known.

The structurally complex nucleic acids are composed of relatively simple primary units: phosphate, the sugars ribose or desoxyribose, and purines and pyrimidines. The sugars as well as the purines and pyrimidines can be synthesized by most organisms and need not be incorporated in the ration. The building materials involved in purine synthesis are glycine, carbon dioxide, ammonia and certain one-carbon compounds such as formate. Folacin is quite generally involved in one-carbon unit metabolism and it appears to be specifically concerned with the synthesis of the purines. Presumably it

is by virtue of this fact that the antifolacin compounds exert their growth inhibitory action. The purine adenine, and to some extent guanine, will when fed to an animal be incorporated into the nucleic acid structure. When adenine is fed both the adenine and guanine in the nucleic acid rises. The nucleoside adenosine and the nucleotide adenylic acid can when fed to the rat become incorporated into nucleic acid adenine and guanine. Under similar conditions guanylic acid is incorporated only into nucleic acid guanine (G. B. Brown, *Ann. N. Y. Acad. Sci.* **60,** *185 (1954)*). By contrast, the free pyrimidines when fed are only slightly incorporated into nucleic acids, while their nucleosides and nucleotides are more extensively incorporated. Thus nucleic acid synthesis might be interfered with by the use of folacin antagonists (to block one-carbon fragment incorporation) or by the use of purine or pyrimidine analogs (to block interconversion reactions and incorporation). One of the more effective analogs available today is 6-mercaptopurine.

The discovery of this compound stemmed from a systematic investigation of purine and pyrimidine analogs (G. H. Hitchings and G. B. Elion, *Ann. N. Y. Acad. Sci.* **60,** *195 (1954)*). These studies showed that the replacement of oxygen by nitrogen or sulfur led regularly to antimetabolites. Thus thiouracil and thiothymine were competitive with uracil and thymine. Thioguanine was effective, but its synthesis was difficult by the older methods.

The isolation of a 6-mercaptopurine (6-MP)-resistant strain of *Lactobacillus casei* (G. B. Elion, S. Singer, and G. H. Hitchings, *Ann. N. Y. Acad. Sci.* **60,** *200 (1954)*) made it possible to show that in the wild form of this microorganism the conversion of adenine into guanine follows two paths: a rapid reaction leading from adenine to hypoxanthine to guanine and a slower reaction following some other path. The mutant resistant to 6-MP cannot utilize hypoxanthine and presumably lacks the mechanism for converting hypoxanthine to guanine. It seems likely then that the action of 6-MP in the wild strain is to block this conversion.

In contrast, H. E. Skipper (*Ann. N. Y. Acad. Sci.* **60,** *315 (1954)*), using mice bearing sarcoma 180 or adenocarcinoma 755, found that the incorporation of isotopically labeled formate into nucleic acids of the tumors and of the intestine was inhibited by 6-MP. It was suggested that hypoxanthine ribotide may be a common precursor of adenine and guanine ribotides.

The effect of some purine analogs on embryonic and regenerating tissues has been studied by S. Bieber, R. Bieber, and G. H. Hitchings (*Ann. N. Y. Acad. Sci.* **60,** *207 (1954)*). Eggs of the frog *Rana pipiens* were incubated with the several purine analogs and rate of development was noted. With 6-mercaptopurine modest retardation of development was noted in eggs taken from well-nourished females. In contrast, eggs from starved females were extremely sensitive, showing not only retarded rate of development but also abnormalities such as microcephalia, poor eye development and aberrant gill formation. The eggs were susceptible only if exposed to the antagonist from the very earliest stages of development. Adenine and adenylic acid gave a partial reversal of these effects. Thioguanine, 8-azaguanine and 2,6-diaminopurine did not affect development. Both 6-mercaptopurine and thioguanine inhibited the rate of regeneration of amputated tadpole tails. Reversal was noted with adenine, adenylic acid and guanylic acid. The effects of the inhibitors were most marked during blastoma formation.

A study of the effect of 6-mercaptopurine on the development of the rat fetus was made by J. B. Thiersch (*Ann. N. Y. Acad. Sci.* **60,** *220 (1954)*). Varying doses of the substance were administered at intervals during the period of pregnancy. The greatest effects were noted when 6-mercaptopurine

was given on the seventh and eighth days of pregnancy—the time of implantation. Large doses given at this time resulted in extensive fetal resorption. Although implantation had occurred, no proper placenta developed. Smaller doses permitted placenta formation but many fetuses were stunted. Many died and were resorbed. The effects on the mother were retarded growth, anemia and diarrhea. Aside from the stunted development of the fetuses, no gross abnormalities were observed. There was no case of hydrocephalus, and the skeletons were not abnormal.

Despite the nonspecific effects of 6-mercaptopurine on embryonic and regenerating amphibian tissue and on rat fetuses, the compound appears to have the property of selectively inhibiting certain tissues. J. J. Biesele (*Ann. N. Y. Acad. Sci.* **60,** *228 (1954)*) showed that mitotic activity of tumors and embryonic tissues was depressed by exposure to 6-MP. These studies were done on tissue cultures and showed that mitosis in embryonic mouse skin was only slightly depressed. Cultures of newborn mouse heart were more drastically affected, while a number of tumors were severely inhibited. Some reversal of this inhibition could be obtained with a variety of purines, nucleosides and nucleotides. Particularly effective were inosinic acid (the nucleoside of hypoxanthine) and adenylic acid. The greatest degree of reversal was obtained with coenzyme A.

Animals carrying certain experimental tumors can be partially protected (survival time extended) by 6-mercaptopurine. This is particularly true of some experimental leukemias. The therapeutic use of 6-mercaptopurine has been particularly directed against human leukemias (*see Nutrition Reviews* **13,** *120 (1955)*).

As a purine analog 6-mercaptopurine should be useful in elucidating the mechanism of biosynthesis of purines, the transformation pathways, and the manner of incorporation into nucleic acids. The effect on living tissues has so far been largely nonspecific. Its effectiveness in controlling human leukemia has shown only some promise.

FLUORINE DEPOSITION IN BODY TISSUES

The small amounts of fluorine in bones and teeth appear to exert considerable effect on the structural properties of these hard tissues. The small amounts required, the difficulty of preparing diets sufficiently low in this element, the analytic difficulties—these are some of the factors which have slowed the development of our knowledge of fluorine metabolism.

S. H. Jackson (*Canad. J. Biochem. Physiol.* **33,** *93 (1955)*) has now investigated the accumulation of fluorine in the body tissues of the rat during the early months of life. Three groups of rats were placed on diets containing 0, 0.5 and 1.0 mg. of fluorine per 100 g. of diet. The animals were one month old at the start of the experiment and were kept on the experimental rations for six months. At monthly intervals successive sets of 5 rats from each of the three groups were killed and the carcasses were analyzed for fluorine. Similar analyses on one month old rats permitted the calculation of the amount of fluorine acquired during the experimental period on each of the three levels of fluorine intake.

Since the basal ration contained a minute amount of fluorine, a small accretion of this element occurred even on diets not supplemented with it. With fluorine supplementation the element accumulated roughly in proportion to the amount fed. On all three diets the total amount of carcass fluorine increased throughout the six-month feeding period. Similarly, carcass fluorine concentration (milligrams of fluorine per

100 g. of body weight) continued to increase, although at a steadily diminishing rate, during the experimental period. The concentration of fluorine in the ash, however, reached a constant value at 2 to 3 months of age in rats receiving 0.5 mg. of fluorine per 100 g. of diet and at 5 months in those receiving 1.0 mg. per 100 g. of diet. The concentrations of fluorine in the whole carcass as well as in the ash increased with the level of dietary fluorine.

A second experiment established the fact that fluorine retention was identical whether the element was fed continually as part of the ration or whether it was administered as a single large dose given once during the week. About 35 per cent of the fluorine was retained during eight weeks of feeding at a level of 1 mg. per 100 g. of ration.

A third experiment tested the effect of high and low calcium intakes on fluorine retention. The total amount retained by the animals was not affected by the dietary calcium levels. However, since the fluorine intake was greater with the high calcium ration, the percentage retention was inversely related to the dietary calcium level and to the total ash content of the carcass. Since in the earlier experiments fluorine accumulated somewhat in proportion to body ash, it may be that the high calcium level of the third experiment interfered with fluorine absorption.

There is much to be learned about the precise functions of fluorine in bones and teeth as well as in soft tissues. More needs to be known about the mechanism of absorption and the influence of other elements such as calcium, magnesium and aluminum upon its metabolic fate.

VITAMIN E AND THE CO-POLYMERIZATION OF PROTEIN AND LIPIDS

The ability to inhibit the oxidation of unsaturated fatty acids and other oxidizable substances is one of the most striking chemical properties of vitamin E and has led biochemists to speculate that the metabolic action of the tocopherols may be related to this property. In reviewing the current status of our information concerning vitamin E action, H. A. Mattill (*Nutrition Reviews* **10,** *225 (1952)*) wrote that, "Perhaps the tocopherols as biologic antioxidants have a protective function in preventing the appearance of tissue-damaging products of oxidation, among them fat peroxides. The various kinds of radiation, cosmic and otherwise, to which animals are exposed, generate peroxidic substances and, like sulfhydryl groups in the aqueous phase, tocopherol, soluble in the fat phase, may be needed to destroy them, vicariously." One evidence of the presence of abnormal fat oxidation *in vivo* is the appearance of abnormal brown pigment in the fatty depots of certain vitamin E-deficient animals. This fatty pigment appears to be related to the ceroid found in the livers of animals with experimental choline deficiency.

The histochemical properties of this pigment are of some interest and support its probable origin from unsaturated lipids. Although it is insoluble in organic solvents, it stains with fat stains even in paraffin sections. It is also stained by acid-fast stains and with the periodic acid-Schiff reaction. In ultraviolet light it possesses a blue-green fluorescence.

W. S. Hartroft (*Science* **113,** *673 (1951)*) was able to produce a similar substance *in vitro* by incubating mixtures of cod liver oil and red blood cells. The study of the *in vitro* production of ceroid was carried further by W. G. B. Casselman (*J. Exp. Med.* **94,** *549 (1951)*) who found that its formation from unsaturated fatty acids was accelerated by the addition of tissues,

blood cells, erythrocytic stroma, or hemoglobin, by emulsification, by increasing the surface of lipid exposed to air, and by increasing the temperature. He suggested that the role of blood cells and necrotic tissues might be both as catalyst and as a surface upon which ceroid may be deposited. The insolubility in organic solvents was attributed to protein binding—a union which in some way frequently inhibited the usual histochemical stains for protein.

This approach has been extended by A. L. Tappel (*Arch. Biochem. Biophys.* **54,** *266 (1955)*). The addition of hemin, hemoglobin, and cytochrome c to mixtures of cod liver oil and casein before shaking in the presence of air all gave large increases in the rate of formation of the yellow-brown pigment and this was attributed to the catalysis of fat oxidation by the iron porphyrin group. Tappel has termed the combination of oxidized lipid and protein co-polymerization. The finding of iron and hemoglobin in the yellow-brown pigment *in vivo* suggests that the *in vitro* observations have physiologic significance. A comparison was made of the rate of co-polymer formation from cod liver oil emulsions in the presence of amino acid nitrogen sources of differing complexity. It was found that the rate was least for free amino acids (glycine), intermediate for mixtures of amino acids and peptides (enzymatic hydrolysates of lactalbumin and casein), and greatest for complete proteins (egg albumin, bovine serum albumin and sodium caseinate). The nitrogen content of the co-polymers varied widely, between 0.7 and 13.3 per cent. The co-polymers formed with cod liver oil and protein had less nitrogen than those formed between linoleic acid and protein.

The action of phenolic antioxidants on a cod liver oil-aqueous caseinate-hemoglobin system was studied. Oxygen uptake was found to be almost completely inhibited by di-tertiary-butyl-para-cresol, propyl gallate, nor-dihydroguaiaretic acid and butylated hydroxyanisole. The inhibition of oxygen uptake by alpha tocopherol was considerably less. It was also found that the formation of co-polymer was inhibited to a much greater extent by the commercial antioxidants than by alpha tocopherol. The apparent weakness of alpha tocopherol in this system might be interpreted as evidence against the thesis which the author wishes to establish. The fact, however, that tocopherols are the only phenolic antioxidants which are readily absorbed by the animal body and are capable of undergoing reversible oxidation and reduction within the body suggests that vitamin E *in vivo* might act as a more effective agent than was indicated in the *in vitro* test system.

The somewhat ill defined nature of the product of these *in vitro* studies makes the translation to the *in vivo* situation difficult. It would seem adequately established that the formation of co-polymers between lipids containing unsaturated fatty acids and proteins is catalyzed by the presence of heme pigments and is inhibited by antioxidants. Tappel suggests that the *in vivo* changes in catalytic and structural protein due to vitamin E deficiency may result directly from the reaction of the products of abnormal unsaturated fatty acid oxidation with protein.

CORTISONE AND THE METABOLIC RESPONSE TO INJURY

Changes in the metabolism of various nutrients are commonly observed after serious injury. Hyperglycemia, glycosuria, lactacidemia, impaired glucose tolerance, ketosis, sodium and water retention, and increased potassium and nitrogen excretion have all been described. The last, in particular, has been the basis for many investigations. Typically, a severely injured individual excretes excessive amounts of

nonprotein nitrogen for three to seven weeks following injury. Fecal nitrogen excretion is unchanged.

Although the increase in urinary nitrogen excretion following injury was described about twenty-five years ago by D. P. Cuthbertson (*Biochem. J.* **24,** *1244 (1930)*), we still are not entirely clear as to its pathogenesis (*Nutrition Reviews* **10,** *195 (1952)*; **11,** *265 (1953)*; **12,** *265, 328 (1954)*). Among the factors which have been considered important in this regard is an increase in adrenal activity. Selye noted many years ago that when animals were exposed to a variety of injurious agents, tissue disintegration, cellular atrophy and necrosis occurred in various organs with the notable exception of the adrenal cortex. On the contrary, the adrenal cortex almost invariably hypertrophied. At the same time, the lipid granules which normally are present in the adrenal cortex were decreased. Not long thereafter this was correlated with an increased secretion of the adrenocortical steroids, measured first by an increase in certain of their metabolic end products in the urine, and, more recently, by an increase in certain of the circulating adrenocortical steroids. It was also shown that many manifestations of the stress reaction are absent in adrenalectomized animals. This was in keeping with an old clinical observation, namely, that vulnerability to exogenous stress is one of the chief characteristics of patients with adrenal insufficiency.

After injury, amounts of adrenocortical hormones are apparently secreted which are capable of causing metabolic upsets when given in otherwise nonstress situations. Since many of the metabolic changes seen after injury are similar to those induced by excess hydroxycortisone or cortisone administration, increase in adrenal activity has been considered important in the pathogenesis of the metabolic reaction to injury. This thesis has gained some support from the findings of roughly parallel increases in urinary nitrogen excretion and apparent adrenocortical hormone secretion in relation to severity of injury—the more severe the injury, the greater the apparent adrenal activity and the greater the nitrogen loss. However, certain studies have demonstrated that the increase in urinary nitrogen excretion after injury can occur in the presumed absence of any concomitant increase in adrenal hormone production. A recent study of this nature has been reported by R. M. Campbell, G. Sharp, A. W. Boyne, and D. P. Cuthbertson (*Brit. J. Exp. Path.* **35,** *566 (1954)*).

These authors conducted a series of experiments in rats to determine, first, whether injury and cortisone administration have similar effects on protein metabolism as judged by urinary nitrogen excretion, and, second, whether adrenalectomized rats, receiving a constant maintenance dose of cortisone, react to injury with an increase in urinary nitrogen excretion similar to that of nonadrenalectomized control animals.

Young adult male rats were offered twice daily a constant amount of food somewhat less than their normal ad libitum intakes. In the first group of experiments, single femoral fractures were produced in some rats by applying a bone-breaking forceps to the surgically exposed femurs. In some of these rats one or two tablets containing 25 mg. of cortisone acetate were implanted subcutaneously at the time of fracture. In other rats without fractures one, two, or three lactose tablets containing either 25 mg. of cortisone acetate or 15 mg. of cholesterol (control) were implanted subcutaneously in various combinations. Finally, in other rats one, two, or three surgical incisions of the type necessary for implantation of the cortisone or control cholesterol tablets were made.

Campbell and her associates noted that following the implantation of 25 mg. of cortisone acetate there was an increase in urinary nitrogen excretion similar in magnitude and shape to that following

femoral fracture whether either was applied alone or along with the implantation of an additional 25 or 50 mg. of cortisone. Most of the response to fracture or implantation of cortisone appeared to result from the necessary incisions. Accordingly, it is not possible to assess definitely the specific contribution of the administered cortisone. While the increase in urinary nitrogen excretion resulting from implantation of a second tablet was similar to that from the first, there was little additional response due to implantation of a third tablet.

In a second group of experiments, femoral fractures were produced in adrenalectomized and sham-operated rats maintained on 0.9 per cent saline and in adrenalectomized and sham-operated rats maintained on oral cortisone (0.8 mg. per 100 g. control body weight daily) and in sham-operated rats with neither oral cortisone nor saline. No increase in urinary nitrogen excretion following fracture occurred in the saline-maintained adrenalectomized rats, but similar responses occurred in the others.

These latter findings are similar to those reported previously by D. J. Ingle, E. O. Ward, and M. H. Kuizenga (*Am. J. Physiol.* **149,** *510 (1947)*). These workers found the same increase in urinary nitrogen excretion after fractures in adrenalectomized rats maintained on a constant small amount of injected adrenocortical extract as in sham-operated controls. No increase was found in adrenalectomized rats maintained on saline. They concluded that the negative nitrogen balance which characteristically develops following injury may require the presence of the adrenocortical hormones but is not caused specifically by the increase in secretion of the cortical hormones which occurs during stress. F. L. Engel (*Endocrinology* **50,** *462 (1952)*), studying urea formation in nephrectomized rats under various experimental situations, has come to a similar conclusion. Similar observations in rats in regard to the occurrence of hyperglycemia, such as that following diethylstilbestrol administration (Ingle, *Am. J. Physiol.* **138,** *577 (1943)*), or traumatic shock (H. Selye and C. Dosne, *Proc. Soc. Exp. Biol. Med.* **48,** *532 (1941)*), have been made.

These data suggest, then, that some of the metabolic changes following injury can occur in the presumed absence of any increase in adrenocortical hormone secretion, provided a certain "maintenance" level of these hormones is provided. This has been termed the "permissive" or "supporting" action of the adrenocortical hormones by D. J. Ingle (*J. Clin. Endocrinol. Metab.* **14,** *1272 (1954)*) and, from a somewhat different point of view, the "conditioning" action of these hormones by H. Selye (*Ibid.* **14,** *122 (1954)*).

Campbell and co-workers call attention to the possibility that in adrenalectomized animals kept on maintenance doses of adrenal hormone accessory adrenal tissue may respond to trauma in the same fashion as intact adrenals, thus allowing a full response to injury. Measurements of steroid secretion after injury by such animals would clarify this possibility.

It should be emphasized that the fact that one can get an increased urinary nitrogen response in the apparent absence of an increased adrenal activity under certain experimental conditions does not mean that the same mechanism obtains in the intact individual in whom there is increased adrenal activity. It is generally agreed that there is an association between the level of cortical hormones secreted and the extent of metabolic response to severe stresses. Depending on the severity of the stress, the "maintenance" level of adrenocortical hormones necessary in adrenalectomized animals for the full metabolic reaction to occur may vary. However, the specific nature of the relationship between the biochemical changes following injury and adrenal activity is not known. It is likely that studies of specific tissue metabolism will be required to establish this.

SELENIUM IN SERUM PROTEINS

Earlier work by McConnell (*see Nutrition Reviews* **6,** *263 (1948)*) showed that when a dose of sodium selenate was injected into lactating rats, selenium appeared in the milk proteins within twenty-four hours. The selenium was distributed among the various protein fractions of the milk in a manner which was roughly proportional to the amount of protein.

An extension of this work by K. P. McConnell and E. J. Van Loon (*J. Biol. Chem.* **212,** *747 (1955)*) has shown that following the injection of labeled selenium into a dog this element was incorporated very rapidly into the plasma proteins. Twenty-four hours after the injection of selenium chloride containing radioactive selenium, the plasma proteins were fractionated by filter paper electrophoresis. Approximately 60 per cent of the radioactivity was equally distributed between both the $alpha_2$- and $beta_1$- globulin fractions. Smaller amounts were found in the other protein fractions.

In another experiment trichloroacetic acid was used to precipitate the selenium proteins collected twenty-four hours after the injection of the radioactive selenium. About 80 per cent of the total selenium in the serum was protein-bound. The combination of the selenium with the protein was of such a nature that it could not be broken by dialysis (K. P. McConnell and B. J. Cooper, *J. Biol. Chem.* **183,** *459 (1950)*). These workers reported that following multiple injections of selenium subcutaneously into dogs, the concentration of this element in the red cells increases with time and that the concentration in the red cells is greater than that in the plasma. Once the injections are stopped, the level of selenium in both the cells and the plasma rapidly decreases.

The concentration of selenium in the plasma protein fractions ranged from 1.5 micrograms per gram for the gamma globulin to 8.1 micrograms per gram for the $alpha_2$-globulin. It is interesting that gold combines with the same plasma fractions as selenium (N. Simon, *Science* **119,** *95 (1954)*). The binding of the gold with these fractions occurs whether the gold is added to the blood *in vitro* or injected intrapleurally. The concentration of selenium in these proteins is much lower than the iron- or copper-combining capacity of some of the beta globulin proteins. The latter proteins can combine with 1.25 mg. of iron or 1.4 mg. of copper per gram of protein (D. M. Surgenor, B. A. Koechlin, and L. E. Strong, *J. Clin. Invest.* **28,** *73 (1949)*). The combination of iron and copper with the proteins can be reversed by changing the pH of the solution.

Although this work does not indicate the manner in which selenium produces its toxic effect, it does, nevertheless, reemphasize the fact that this material can very rapidly combine with a large number of different proteins. Whether this is an exchange reaction whereby the sulfur in certain amino acids is replaced by selenium has not been determined.

It would be worth while to repeat the plasma protein studies on dogs that have been chronically poisoned with selenium. Since such animals develop a severe anemia (M. Rhian and A. L. Moxon, *J. Pharmacol. Exp. Therap.* **78,** *249 (1943)*), there might be a relation between the concentration of the selenium in the red cells and the degree of anemia.

NOTES

Letter to the Editor

Dear Sir:

Perhaps your readers would like to hear something of nutritional problems on the archipelago of Sangir, Indonesia, and particularly of how much I appreciate receiving *Nutrition Reviews*. I am the only physician in this group of islands, the most northern outpost of Indonesia (almost in the Philippines), and have a population of 120,000 to look after. *Nutrition Reviews* is about my only scientific contact with the recent nutrition literature and you can imagine it is read and reread.

I studied the important article of Dr. Hegsted, "False Estimates of Adult Requirements" (*Nutrition Reviews* **10,** *257 (1952)*). Is it wrong to state that our recommendation of a certain protein intake (1 g. protein per kilogram of body weight) is false too? This is of outstanding importance for the food situation in Asia.

In my "Mother and Child Health" Centre I am collecting very rough anamnestic and clinical data, trying to correlate dietary intake and health of mother and infant. I must confess that a theoretically very poor diet (mother's milk, and, by mother or grandmother, prechewed tubers) provides a healthy and gay-hearted infant, indeed a little too fat and flabby, but strong enough to resist childhood diseases (malaria included), as his 4 elder brothers and sisters demonstrate.

It is a sound and even comforting statement to conclude that, as the population is not withering away, the healthy adult must be in slight positive protein balance over the long run.

Another conclusion from Dr. Hegsted's study is that the well known habit of the slamatan in Java and Sangir—ceremonial superfluous eating parties with much meat but no vegetables—is inefficient, for the sudden and strong rise in protein intake results in considerable loss of the protein, accentuated by the absence of other nutrients (vegetables) needed for efficient absorption and utilization.

Consequently we will have to guide these people in the direction of more equally distributed food intake. However, social habits and economic conditions (harvest) may make it impossible.

Reprints of diet and iron resorption are important to me as I am interested in features of the stippled pigmented tongue clearly pictured in figure 151 in "Perspectives in Human Malnutrition" (J. and T. Gillman, *Grune & Stratton, New York (1951)*). Contrary to the findings of Gillman, I find the initial symptoms too in 8 year old schoolboys. I know all the stages, have seen the development during pregnancy, and believe the symptom is irreversible. I should like to ask if the nutritional iron deposition in the liver is irreversible too. A remarkable study in this field is from W. Masshoff and E. Waldschütz (*Virchows Arch. f. path. Anat.* **320,** *618–630 (1951)*). The importance of this topic is lucidly demonstrated in "Hemoglobin and Body Iron in Protein Deficiency" (*Nutrition Reviews* **12,** *341 (1954)*).

The tongue symptom is not of acute value for the person concerned. It indicates only that something has happened. Most of the people inform us that this was the fact in their earlier youth. Every deficiency symptom has to be considered in relation to its acute value. This is the fact with the parotid swelling and liver enlargement, too.

Whenever there is not an acute value frequent occurrence in a community has a real meaning for this community: at some time there may be some deficiency which causes disease and consequently death or recovery with some scar. So I consider frequent occurrence of liver enlargement as a scar of protein deficiency in the community and the stippled pigmented tongue indicative of impaired iron metabolism at a certain age or circumstance (pregnancy).

The practical value of certain community

deficiency signs ("scars") is that you can pick out rather quickly the most affected villages and concentrate your exploration of the causes and eventually nutrition teaching or disease-controlling measures.

Your conclusion that dietary protein deficiency does not alter resistance or physiologic response of the young rat to experimental tuberculosis is also comforting, and in accord with the findings of Dr. B. van Vliet in the Dutch province Groningen, where the frequency of T.B.C. rose equally in town and country districts and nutrition was not disturbed. Infection through unhygienic conditions and growing mobility of the community were the most important factors ("Medische ervaringer in Nederland tijdens de bezetting 1940–1945," J. Boerema, editor, *p. 431, J. B. Wolters, Groningen (1947)*).

The review of maternal diet and fetal liver (*Nutrition Reviews* **12,** *104 (1954)*) was very interesting to me. Is it true that only the weight of the liver depends on the nutrition state of the mother? It would provide us with a nice and simple possibility of weighing this accurately and (1) confirm the results of Smith, Worcester and Burke, (2) indicate undernutrition as a possible cause of the abortion or prenatal death in the concerned case.

As a matter of fact I see many abortions, premature births and prenatal deaths here, which are supposed to be related to a poor nutritional state of the mother. I wrote a two-page guide on mother and infant feeding in Sangir which has been given to about 3000 mothers via the Mother and Child Health centres. Eating fish, green beans (katjag idju = Phaseolus radiatus) and vegetables from early childhood (7 months) is stressed.

We have distributed 15,000 kg. skim milk powder to almost 4000 pregnant and nursing mothers, spread over the whole area, to call their attention to the importance of nutrition for mother and child taught in the leaflet. In our hospital there is much interest. In my area there are 6 graduate midwives and several assistant midwives working; they are helping to propagate this topic. It is a hard job to draw attention from the curative to the preventive corner. However, in modern obstetrics there is already some tradition.

You see your sending of literature to me is stimulating my field work although I am not yet able to condense one simple subject in an article. My first task is to work for the 120,000 inhabitants of this archipelago. The hospital with 1600 admissions requires much attention, as does the Health Service administration, the pharmaceutical supply and the budget, for which I am personally responsible due to lack of trained personnel.

I hope that you will continue to send me the *Nutrition Reviews*, as they are my scientific guidance in the field of nutrition.

D. M. Blankhart
Taruna
Sangir, Indonesia

Tributes to Scientist

Friends and colleagues of Professor Albert G. Hogan arranged a dinner in his honor which was held on the University of Missouri campus, Columbia, May 21. Dr. A. H. Smith of the Wayne University College of Medicine was the principal speaker, and in behalf of friends and former students Dr. N. B. Guerrant of Pennsylvania State University presented a portrait of Dr. Hogan to the University of Missouri. The Hales and Hunter Company of Chicago presented a graduate fellowship to be known as the Hogan Fellowship in Nutrition. At the June 8th Commencement Dr. Hogan was made Professor Emeritus.

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